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EXPERIMENTAL THERAPEUTICS

Lichtheimia corymbifera Colonization Leading to Pulmonary Infection Can Be Prevented with Liposomal Amphotericin B in a New Murine Model

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ABSTRACT The incidence of pulmonary mucormycosis is constantly increasing, especially in hematological patients staying in high-efficiency particulate air-filtered rooms. Pulmonary inhalation of spores may occur outside the hospital, leading to invasive disease once patients received chemotherapies. We developed a new pulmonary mucormycosis mouse model mimicking the expected pathophysiology in human to study antifungal drugs. Naive mice were inoculated intratracheally with *Lichtheimia corymbifera* spores. After 3 days, mice received corticosteroids and cyclophosphamide and secondarily developed the disease, while only 5% of the initial inoculum was present in the lungs at day 3. Lung colonization with *L. corymbifera* spores in immunocompetent mice can last at least 44 days. Antifungal drug was administered the day of immunosuppression. Injection of a single 15 mg/kg of body weight dose of liposomal amphotericin B significantly improved survival and pulmonary fungal burden compared with controls, whereas 80 mg/kg oral posaconazole did not. These results show that a unique dose of liposomal amphotericin B offers a real potential decolonization treatment to prevent infection in our mouse model of *L. corymbifera* lung colonization followed by lung infection.

KEYWORDS antifungal agents, invasive fungal disease, *Mucorales*, mucormycosis, prophylaxis

Despite marked progress in the diagnosis and treatment of invasive fungal diseases (IFDs), prognosis of mucormycosis remains very poor, with an overall mortality of 50% (1). This rate can reach 80% to 100% in patients with prolonged neutropenia (2). Since the primary route of human infection is inhalation of airborne spores, sinus and pulmonary involvement are the most prevalent sites of infection. In a French series of 101 mucormycosis cases, lung localization occurred in 28% of the cases, mostly in patients with hematological malignancies (3). Although *Lichtheimia corymbifera* was the second most common pathogen after *Rhizopus oryzae* responsible for mucormycosis, it is known to be more associated with pulmonary involvement and hematological malignancies (4). In the hematological setting, mucormycosis accounted for 7.2% of IFD, with a higher prevalence of pulmonary mucormycosis (5). However, the incubation period between environmental exposure to spores and development of mucormycosis is unknown, and disease may occur in patients staying for days or weeks in an air-filtrated room. By analogy with *Pneumocystis jirovecii* pneumonia, an unknown length of colonization could precede the invasive disease (6). Spores could also remain

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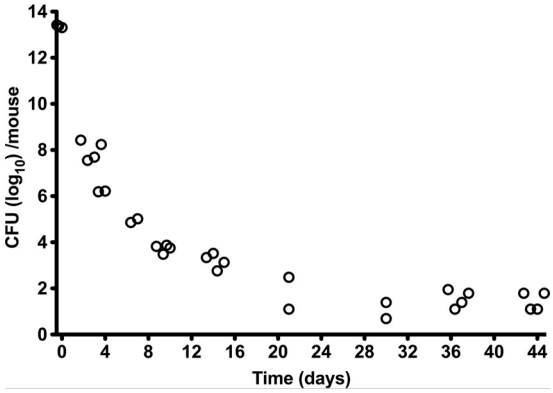

FIG 1 Kinetics of *L. corymbifera* clearance in the lungs of immunocompetent BALB/c mice. A total of 35 BALB/c mice were inoculated intratracheally with 10^6 spores of *L. corymbifera*/mouse. Groups of two mice were euthanized at various times postinoculation. CFUs in the lungs were then enumerated. Each circle represents a mouse.

dormant in the lung inside granuloma and reactivate once the patient is exposed to immunosuppressive therapies, as it has been shown for other fungi, such as dimorphic fungi (7). Because controlling the inhalation of spores is difficult, efficient mucormycosis prevention is crucial.

Posaconazole (PCZ) is currently indicated as IFD prophylaxis during graft-versus-host disease in hematopoietic stem cell transplant (HSCT) recipients and in patients undergoing heavily cytotoxic intensive chemotherapy (8). However, several cases of mucormycosis have been described, which are linked to insufficient PCZ plasma concentrations or due to PCZ resistance (9, 10). In addition, this azole provides numerous drug-drug interactions due to inhibition of several cytochrome P450 isoenzymes (11). Another antifungal drug, liposomal amphotericin B (L-AmB), is the current recommended first line for mucormycosis treatment (12). It could be an alternative for PCZ as mucormycosis prophylaxis. Since mouse models are the gold standard to study IFD, they can help to test the efficacy of antifungal drugs (13).

Therefore, we designed a pulmonary model of *L. corymbifera* colonization that includes a period of colonization followed by an immunosuppression period to develop the disease. This model was then used to test antifungal drugs, namely, PCZ and L-AmB, as decolonization treatment.

RESULTS

Lung colonization after *L. corymbifera* intratracheal inoculation in immunocompetent mice. After intratracheal inoculation of 10^6 spores/mouse, the number of CFUs at 2 hours postinoculation in the lungs of mice was $6.3 \times 10^5 \pm 0.6 \times 10^5$ per mouse, representing 63% of the initial inoculum. Pulmonary fungal cultures at day 3 postinoculation (D3pi) were positive, with a mean of $3.1 \times 10^3 \pm 1.3 \times 10^3$ CFU per mouse, representing 5% of the initial inoculum. However, fungal cultures of lungs remained positive 44 days after exposure, with a mean of 4.5 ± 1.7 CFU (Fig. 1).

Timing of immunosuppressive drug administration to induce pulmonary mucormycosis. When the immunosuppressive drugs were administered at D4pi, the mortality rate was 66.7% at D21pi (Fig. 2). To reach the endpoint of 100% mortality at D21pi, cortisone acetate and cyclophosphamide were administered to groups of 8 mice a day earlier, i.e., at D3pi. The mortality rate of infected immunosuppressed mice was 100% at day 18. Since the mice started to die after D10pi, a histopathological examination of the lungs was performed at D9pi. Lungs showed heterogenous profiles of infection, from swollen spores to hyphae invading the lung tissue (Fig. 3). D9pi was then chosen to assess fungal burden in the lungs after decolonization treatment.

Decolonization efficacy in the mouse model. The efficacy of a single and unique dose of L-AmB and PCZ as decolonization treatment was determined using survival

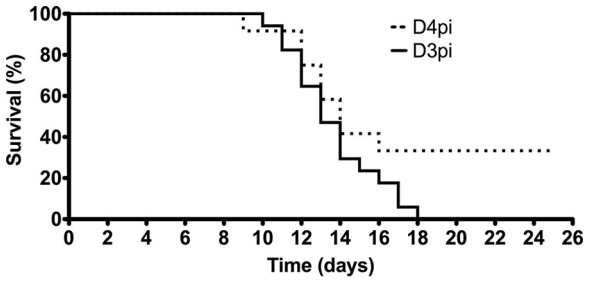

FIG 2 Survival curves of BALB/c mice to determine the day of immunosuppression. A total of 28 BALB/c mice were inoculated intratracheally with 10^6 spores of *L. corymbifera*/mouse. Then, 12 mice were immunosuppressed at day 4 postinoculation (D4pi) and 16 mice at D3pi, with 500 mg/kg cortisone acetate and 200 mg/kg cyclophosphamide. Immunosuppressive drugs were then administered every 5 days.

curves (Fig. 4) and quantification of fungal tissue burden at D9pi (Fig. 5). L-AmB significantly improved survival compared with placebo (mean survival of 20 days versus 13 days; $P = 0.0008$) (Fig. 4A). The median survival time was similar between PCZ and controls ($P = 0.097$) (Fig. 4B). A single administration of L-AmB resulted in a significant decrease in pulmonary fungal burden compared with controls (2.1 $\pm$ 0.2 versus 3.1 $\pm$ 0.8 $\log_{10}$ of spore eq/mg of lung; $P = 0.043$) (Fig. 5A), whereas PCZ did not (3.7 $\pm$ 1.8 versus 2.8 $\pm$ 0.7 $\log_{10}$ of spore equivalents/mg of lung; $P = 0.748$) (Fig. 5B).

DISCUSSION

We developed a mouse model of primary pulmonary colonization by *Mucorales* resulting in invasive disease after immune system disruption. Our results showed that *L. corymbifera* could persist up to 44 days in the lungs of immunocompetent mice. The model was used to test fungal decolonization at the same day of immunosuppression with a single and unique dose of antifungal drug. Systemic L-AmB may be a more effective decolonization treatment than oral PCZ in immunosuppressed mice.

The originality of our model relies on fungal colonization resulting in invasive disease when mice are immunosuppressed. As in humans, mice eliminate spores of fungi when inhaled. Early elimination is mainly due to phagocytosis and mucociliary elevator (14). Even a low quantity of spores colonizing the lungs is able to germinate and invade lung parenchyma after immunosuppression. No experimental models of mucormycosis in the literature used this approach that could yet be relevant in human

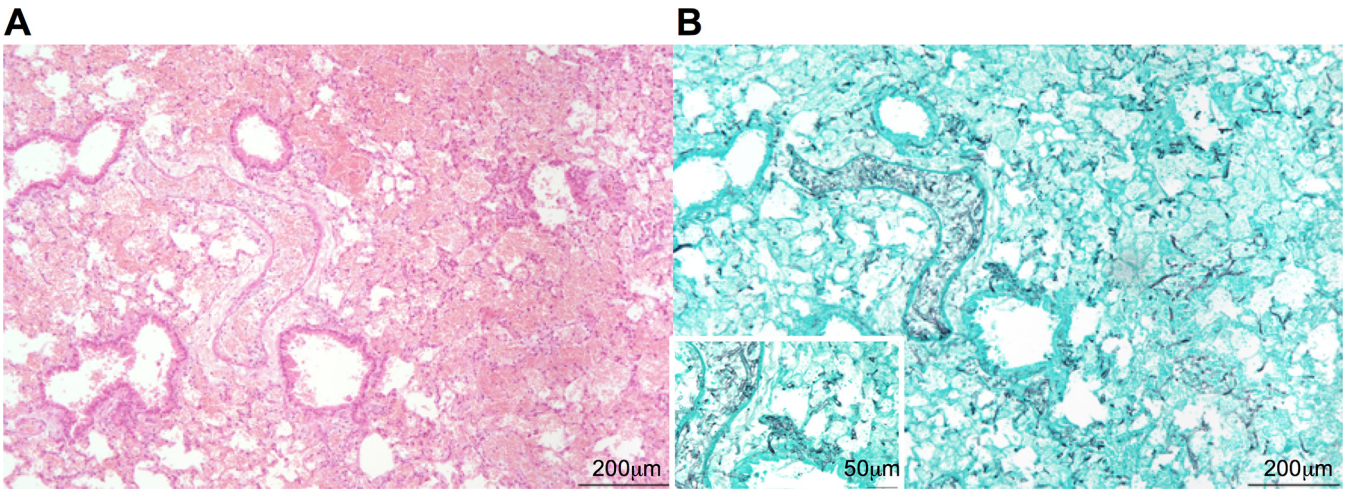

FIG 3 Histopathological examination of a mouse lung infected by *L. corymbifera* at day 9 postinoculation. Three BALB/c mice were inoculated intratracheally with 10^6 spores of *L. corymbifera*/mouse and immunosuppressed at day 3 postinoculation with 500 mg/kg cortisone acetate and 200 mg/kg cyclophosphamide. Pictures of the more damaged lungs are shown. Diffuse hemorrhages of lung parenchyma and necrosis of alveolar epithelium caused by *L. corymbifera* (A, hematoxylin-eosin staining) and fungal hyphae invading the lung parenchyma and the vessels were observed (B, Grocott's methenamine silver staining).

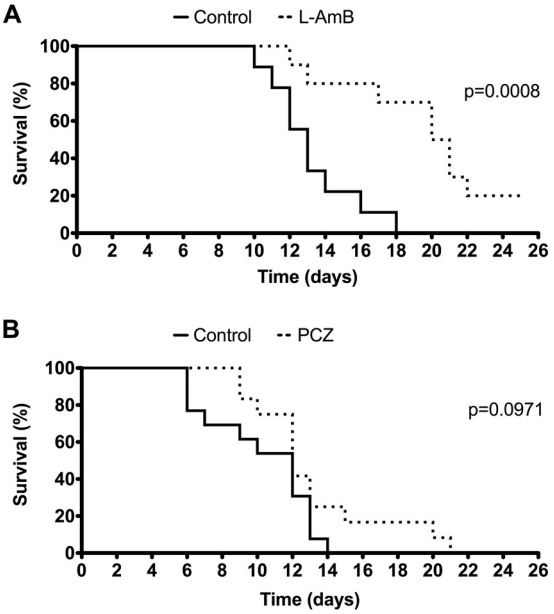

FIG 4 Survival curves of BALB/c mice colonized by *L. corymbifera* secondarily immunocompromised. All mice in this experiment were inoculated intratracheally with 10^6 spores of *L. corymbifera*/mouse and immunosuppressed at day 3 postinoculation with 500 mg/kg cortisone acetate and 200 mg/kg cyclophosphamide. (A) A total of 12 mice were treated with 15 mg/kg intraperitoneal liposomal amphotericin B (L-AmB) and 12 control mice with 5% intraperitoneal glucose serum. (B) A total of 12 mice were treated with 80 mg/kg oral posaconazole (PCZ) on the day of immunosuppression and 12 control mice with sterile water. Groups were compared using log-rank test.

pathophysiology of mucormycosis. In the literature, all other mouse models have systematically tested prophylaxis in mice that had been immunosuppressed before intratracheal (15) or intravenous inoculation (16, 17). In our model, mice were inoculated before immunosuppression. In the literature, prophylactic treatments are injected a few days before fungal inoculation at repeated doses. For example, azoles were given 2 days before and the day of inoculation in the Gebremariam et al. study (15). In another study, PCZ and deoxycholate AmB were injected 2 days before, 1 day before, and the day of inoculation (17). Caspofungin and deoxycholate AmB were given 1 day

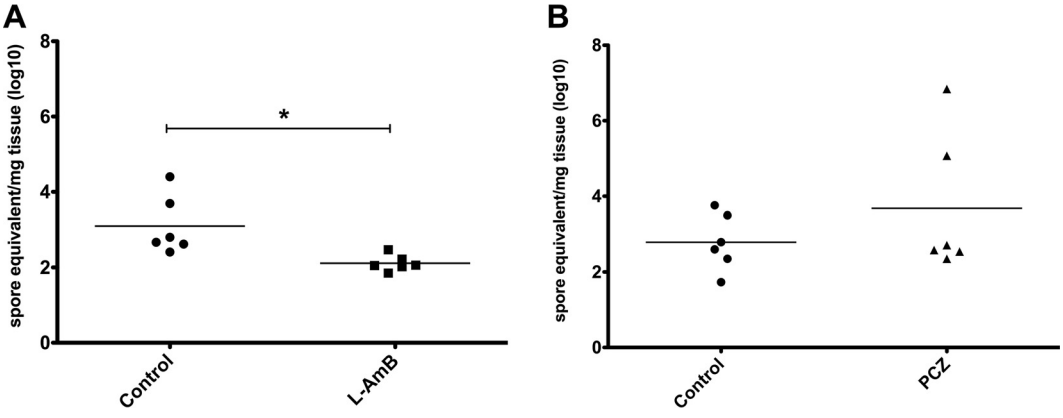

FIG 5 Comparative measurement of pulmonary fungal tissue burden at day 9 postinoculation. All BALB/c mice in the experiment were intratracheally inoculated with 10^6 spores of *L. corymbifera*/mouse, and then immunosuppressed at D3 postinoculation with 500 mg/kg cortisone acetate and 200 mg/kg cyclophosphamide. (A) Six mice were treated with 15 mg/kg intraperitoneal liposomal amphotericin B (L-AmB) and 6 control mice with 5% intraperitoneal glucose serum. (B) Six mice were treated with 80 mg/kg oral posaconazole (PCZ) on the day of immunosuppression and 6 control mice with sterile water. Results are expressed as spore equivalent (eq) of *L. corymbifera* DNA per mg of tissue relative to the organ weight. Groups were compared using Mann-Whitney U test. $^*P = 0.004$.

before and the day of inoculation in the study of Ibrahim et al. (16). In our model, antifungal drugs were used as a decolonization treatment at a single and unique dose the day of immunosuppression and not as prophylaxis. Prophylaxis is used at repeated doses before infection to avoid infection, whereas decolonization is used only once after spore inoculation to eradicate spores before germination. Indeed, the duration of incubation of *Mucorales* spores in patients is unknown. Environmental investigation for the presence of spores is often negative in areas that are protected by air filtration systems (18). As *Mucorales* produce airborne spores, patients' contamination by spores may occur outside the hospital before immunosuppressive treatment. *Mucorales* spores have recently been shown to remain latent in zebrafish granuloma (19). Other molds, such as *Aspergillus* spp., could also remain latent (7, 20). Currently, there is a lack of noninvasive techniques to find *Mucorales* colonization in the lung of patients before administration of immunosuppressive regimen, although *Mucorales* reactive T cells could be a promising tool to investigate mold environmental exposure (21). Besides, even with invasive procedure and with the marked improvement in molecular tools, the diagnosis of mucormycosis remains difficult (22–24). Those molecular tools require thorough standardization, optimization, and validation before considering using them routinely in microbiology laboratories (25). As screening for *Mucorales* pulmonary colonization in patients at higher risk is not yet an option, antifungal decolonization could be useful to avoid germination of persisting spores.

Spores of *L. corymbifera* persisted in the lungs of naive mice at least 44 days after inoculation. However, pulmonary clearance may depend on the fungal species studied. Waldorf et al. have shown complete clearance of *Rhizomucor pusillus* spores after 30 days in immunocompetent Swiss albino mice inoculated intranasally with 5×10^6 spores/mouse (26). In addition, Leleu et al. reported faster elimination of another mold, *Aspergillus fumigatus*, in BALB/c mice only 10 days after inoculation with an aerosol of 3×10^8 to 5×10^8 conidia/ml (20). However, none of these models have considered seeding the lungs in their entirety to assess fungal colonization by culture. Therefore, we cannot exclude that spores were still present in the lungs. This may also explain the difference in fungal pulmonary clearance observed in our model.

The faster fungicidal activity of L-AmB compared with PCZ on *L. corymbifera* may explain the better survival in mice treated with L-AmB. AmB was more rapidly fungicidal, leading to 95% killing as early as 6 h and 99.9% killing at 24 h of *Rhizopus* spp. and *Mucor* spp. *in vitro* (27). Comparatively, PCZ showed less than 70% killing at 6 h and 99.9% killing at 48 h of the same strains (27). In addition, postantifungal effects of AmB and PCZ were studied *in vitro* with *A. fumigatus*. AmB provided a 10-fold longer postantifungal effect than that of PCZ (7.5 h $\pm$ 0.70 h versus 0.75 h $\pm$ 0.35 h), suggesting that L-AmB may have a longer postantifungal effect on *L. corymbifera* (28). This pharmacodynamic property of antifungals has been little studied against *Mucorales*. It has been shown that the postantifungal effect was dependent on *Mucorales* species and on AmB concentration (29).

To the best of our knowledge, only one experimental study was dedicated to prophylaxis with L-AmB treatment (30). This study used nebulized L-AmB in a mouse model of *R. oryzae* pulmonary infection. Outbred mice were immunosuppressed and then infected intratracheally with spores. This study failed to demonstrate the efficacy of nebulized L-AmB started 2 days before infection and continued for 5 days after infection in terms of survival compared with untreated mice. Two other studies have used deoxycholate AmB as prophylaxis (16, 17). These studies showed that two or three doses of deoxycholate AmB given before inoculation reduced fungal load and increased survival. They cannot be compared with ours since the fungal species, the timing of infection and immunosuppressive drugs administration, and the route and duration of treatment are different.

In conclusion, systemic administration of L-AmB offers a real potential as decolonization treatment to prevent the occurrence of mucormycosis in our mouse model of *L. corymbifera* colonization prior to immunosuppression followed by lung infection. In addition, there is no known interaction between chemotherapy and L-AmB, which

could be safely administered the same day as chemotherapy. Assessing other *Mucorales* species in this model could be of interest.

MATERIALS AND METHODS

Ethical statement. Experimental protocols have been approved by the local Comte Poitou-Charentes ethics committee and have been registered by the French Ministry of Higher Education and Research (number 2015110516595628). All investigations were performed in accordance with prevailing regulations regarding the care and use of laboratory animals from the European Commission (directive 2010/63/EU).

***Mucorales* strain and preparation of the inoculum.** The same clinical isolate of *Lichtheimia corymbifera* from the Pasteur Institute strain collection (Centre national de référence des Mycoses invasives et antifongiques [CNRMA] 2011.1047, Paris, France) was used for all experiments (14). This strain comes from a deep pulmonary sampling in a cancer patient. MICs for AmB and PCZ were 0.5 and 0.25 mg/liter, respectively. Aliquots of *L. corymbifera* spores were stored at −80°C. After being thawed, an aliquot was cultured on potato dextrose agar (PDA) tubes (Becton, Dickinson, Le Pont de Claix, France). After 6 days of culture, a subculture was performed on 75-cm^3 flasks containing 15 ml of PDA. After additional 6 days, the spores were collected by flooding the flask with 10 ml of phosphate-buffered saline (PBS; pH 7.4; Gibco, Life Technologies, Marly-le-Roi, France) supplemented with 0.05% Tween 80 (Sigma, Saint Quentin Fallavier, France) (PBS/Tween 80) to avoid spore aggregation. The suspension was filtered with a 11-μm nylon filter (Millipore, Carrigtwohill, Ireland) to remove hyphal elements, and then centrifuged at 3000 $\times$ *g* for 3 min at 4°C. The supernatant was removed and the pellet resuspended in 3 ml of PBS/Tween 80 to obtain a spore suspension. The spores were counted using a Malassez hemocytometer. The suspension was then diluted in PBS/Tween 80 to obtain the inoculum suspension of 2.10^7 spores/ml. The inoculum suspension was kept at +4°C for a maximum of 24 h.

Mouse model development. Congenic male BALB/c mice (n = 132), 7 to 9 weeks old, and 18 to 20 g (Janvier Labs, Le Genest Saint Isle, France) were used for all the experiments and housed under sterile conditions throughout the experiments. Naive mice were intratracheally inoculated using a blunt-end, 24-gauge needle (Dutscher, Brumath, France) with a solution containing 10^6 spores/mouse in 50 μl of PBS/Tween 80 following a short anesthesia with isoflurane (Forene, Rungis, France), as previously described (14, 31).

The model was developed following three steps.

Step A. Lung colonization after *L. corymbifera* intratracheal inoculation in naive mice. We first checked the inoculum intratracheally delivered to the lungs of seven naive mice from three separate experiments, sacrificed 2 hours after inoculation. Then, the lower respiratory tract colonization was assessed by lung culture at different times postinoculation up to 44 days (n = 2 per time). Since spores were not able to germinate in naive mice, they are not damaged by tissue grinding, contrary to hyphae (16). Mice were sacrificed using pentobarbital (Vetoquinol, Magny-Vernois, France). The lungs were then removed and homogenized using a tissue grinder (Polytron PT 1600 E; Kirematica AG, Luzern, Switzerland) at 15,000 rpm for 1 min in 1 ml of sterile water. The whole homogenate was then plated onto Sabouraud chloramphenicol medium (SC) plates with 200 μl/plate. The fungal CFUs were enumerated after 24 to 72 h of incubation at 30°C.

Step B. Immunosuppression of mice. After a lag time of 3 to 4 days, mice were immunocompromised with cortisone acetate combined to cyclophosphamide. This combination was commonly used in mucormycosis animal models (15, 31–35). Corticosteroids are used to suppress pulmonary alveolar macrophage function. Cyclophosphamide is used to induce granulocytopenia (32). Groups of 6 mice were immunocompromised at D4pi and groups of 8 mice at D3pi. Experiments were performed in duplicate. Cortisone acetate (Sigma) was dissolved in PBS containing 0.01% Tween 20 (Sigma) to obtain a 50 mg/ml solution, and cyclophosphamide (Sigma) was dissolved in PBS (40 mg/ml). Both drugs were administered intraperitoneally (IP) every 5 days at 500 mg/kg and 200 mg/kg, respectively (15, 31–35). All immunosuppressive drugs were prepared at the day of use. The lag time between inoculation and immunosuppression was defined with survival curves. The endpoint was the development of infection in all immunocompromised mice within 21 days following the injection of immunosuppressive drugs. This time point was arbitrarily chosen as it allowed the development of an acute infection and not a chronic one. The mice were euthanized when they reached the limit points, i.e., weight loss of more than 20% compared with the average weight, passive position in environment, or prostration, or limb palsy, or impossibility to feed.

Step C. Assessment of lung invasive infection. A histopathological examination was performed to ensure invasive disease and presence of hyphae in the parenchyma at D9pi. Lungs of three control mice were aseptically collected and fixed in 60 ml of 10% neutral buffered formalin (VWR Chemicals, Fontenay-sous-Bois, France) for 24 h. They were then immersed in 70% ethanol and embedded in paraffin. Four-micrometer sections were cut in the middle of the paraffin block and stained with hematoxylin and eosin to describe histologic lesions and Grocott methenamine silver for the detection of fungi.

Decolonization assessment in the model. The highest doses of L-AmB and PCZ described in the literature were tested, i.e., 15 mg/kg and 80 mg/kg, respectively (14, 31). The antifungal drugs were prepared at the day of use. L-AmB (Gilead Sciences, Boulogne-Billancourt, France) was diluted in sterile water according to the manufacturer's recommendations. The final concentration of 1.5 mg/ml was obtained by adding sterile 5% glucose serum and was then administered as a single and unique IP injection (200 μl) the day of immunosuppression. Infected untreated control mice received sterile 5% glucose serum as an IP placebo. PCZ oral suspension (Merck, Lyon, France) was diluted 1:2 in sterile water

(20 mg/ml), and 100 μl was given by oral gavage as a single and unique dose the day of immunosuppression. Infected untreated control mice received sterile water as an oral placebo.

Then, the lung infection was assessed by survival curves up to 21 days and lung fungal load measured by quantitative PCR (qPCR) at D9pi. qPCR was used to determine fungal load since tissue fungal burden determined by qPCR but not CFU correlates with disease progression (16). *L. corymbifera* fungal burden in tissue was determined in 3 mice per group by real-time qPCR on lungs. Experiments were performed in duplicate. After collection, lungs were stored at −80°C before use. After thawing, the sample was homogenized in a milling bowl containing 5 ml of sterile water (Fresenius Kabi, Sèvres, France) and 3-mm-diameter steel beads (Nextadvance, New York, USA). The bowl was placed in a Retsch MM 400 vibratory mill (Retsch, Haan, Germany) programmed for 30 movements per second for 2 min 30 seconds. Then, an external prelysis was carried out with a Roche MagNa pure bacteria lysis buffer kit (Roche Diagnostics, Meylan, France). This protocol was as follows: 200 μl of buffer added to 200 μl of crushed material and 40 μl of proteinase K were placed at 65°C for 3 h and then mixed and placed at 100°C for 10 min. Finally, a chemical lysis was performed by a MagNA pure compact instrument automatic extractor (Roche) using 400 μl of the prelysis sample. At the end of the automatic extraction process, 100 μl of eluate was obtained. DNA amplification was performed on a StepOnePlus system (Thermo Fisher Scientific, Illkirch, France) using primers and probes of 18S ribosomal DNA (rDNA) from *L. corymbifera* (36). Oligonucleotide sequences were as follows: (i) sense amplification primer (or forward primer), 5′-CACCGCCCGTCGCTAC-3′; (ii) antisense amplification primer (or reverse primer), 5′-GCAAAG CGTTCCGAAGGACA-3′; and (iii) hybridization probe, 5′-FAM-ATGGCACGAGCAAGCATTAGGGACG-NFQ-MGB-3′. The thermal cycling conditions were as follows: denaturation at 95°C for 10 min and then 15 cycles of 15 s at 95°C and 1 min at 60°C. Each 25-μl qPCR mix consisted of 10 μl of DNA, 100 nM sense and antisense primers, and TaqMan universal PCR master mix (Applied Biosystems, Thermo-Fisher Scientific). The fluorophore used was 6-carboxyfluorescein (FAM), and the dark quencher used was black-hole quencher 1 (BHQ1; Eurogentec, Angers, France). The reactions were performed in 0.1-ml MicroAmp 96-well plates (Applied Biosystems). The fungal burden was reported as spore equivalent (eq) of *L. corymbifera* DNA per mg of lung tissue (32).

Statistical analysis. Statistical analysis was performed using Prism 5 software (Graph Pad, La Jolla, CA, USA). Quantitative variables were expressed as mean ± standard deviation or median (minimum – maximum). Differences in survival rates between groups were assessed using log-rank tests. To compare lung fungal loads obtained by qPCR, Mann-Whitney U nonparametric tests were used. Statistical significance was retained if P values were <0.05.

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